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(71) Applicant: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).

(72) Inventor: PASEK, Mark, P. ; 151 G Street, South Boston, MA 02127 (US).

(74) Agents: HALEY, James, F., Jr. et al.; Fish & Neave, 875 Third Avenue, New York, NY 10022-6250 (US).

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(57) Abstract

The present invention relates to methods for amplifying isolated nucleic acid sequences, particularly DNA sequences, whose nucleotide sequences are unknown. The invention also relates to oligonucleotide primer pairs that may be employed in the amplification methods of the present invention.

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OLIGONUCLEOTIDE PRIMER PAIRS FOR SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHICH EMPLOY THEM

TECHNICAL FIELD OF INVENTION

The present invention relates to methods for amplifying isolated nucleic acid sequences, particularly DNA sequences, whose nucleotide sequences are unknown. The invention also relates to oligonucleotide primer pairs that may be employed in the amplification methods of the present invention.

BACKGROUND ART

The study of genes and the DNA which encodes them is, in some instances, limited by the amount of starting material available for analysis. For example, the successful isolation of a single copy chromosomal gene in the human genome requires the painstaking process of creating a human genomic library and screening millions of genomic clones. Such a screening process would undoubtedly utilize hybridization techniques that employ one or more oligonucleotides. These oligonucleotides would be designed based on some known partial DNA sequence of the gene or on a known partial amino acid sequence of the protein encoded by the desired gene. If and when the clone containing the desired gene is finally isolated, the clone must then be replicated before a useful quantity of the gene is

obtained. If the genomic library is contained in a vector in which the production of large quantities of the gene is not practical (in a bacteriophage, for example), the gene, once isolated, must be removed from the first vector and subcloned into a more suitable second vector (such as a plasmid). This entire procedure would also be necessary for the isolation of cDNA clones which correspond to rare messenger RNAs (mRNAs) from a cDNA library.

The recent development of polymerase chain reaction (PCR) technology has, in certain circumstances, overcome many of the above difficulties [K. B. Mullis and F. A. Faloona, "Specific Synthesis of DNA In Vitro Via a Polymerase-Catalyzed Chain Reaction", Methods Enzymol., 155, pp. 335-50 (1987); R. K. Saiki et al., "Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia", Science, 230, pp. 1350-54 (1985)]. PCR amplification involves the synthesis of two oligonucleotide primers that are complementary to specific sequences that flank the DNA segment to be amplified. Initially, the double-stranded DNA is heat denatured into single strands. The primers are then added and allowed to anneal to their complementary sequences. The primers hybridize to opposite strands at opposite ends of the target DNA sequence. DNA replication is then effected by the addition of DNA polymerase and proceeds across the region between the primers, effectively doubling the amount of that DNA sequence. Since the replicated sequences are also complementary to the added primers, each successive round of replication essentially doubles the amount of DNA synthesized in the previous cycle. This results in the exponential accumulation of the specific sequence to be replicated. Therefore, PCR

is a technique enabling those of skill in the art to obtain useful quantities of specific, known DNA sequences heretofore available only in minute amounts.

A main drawback associated with the PCR technique was the thermolability of the Klenow fragment of E.coli DNA polymerase I used to catalyze the DNA replication. Because heat denaturation is required to separate the newly synthesized strands at each cycle of replication, fresh Klenow fragment had to be added during each cycle. Such a process was tedious, error-prone and costly.

More recently, a thermostable DNA polymerase has been purified from the thermophilic bacterium Thermus aquaticus [European patent application 258,017; R. K. Saiki et al., "Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase", Science, 239, pp. 487-91 (1988)]. This "Taq" polymerase is stable at temperatures up to 95°C and is thus ideally suited for PCR. The use of Taq obviated the need for repeated additions of DNA polymerase and paved the way for automated PCR [DNA Thermal Cycler, Perkin-Elmer/Cetus, Norwalk, CT].

PCR has been used to generate cDNAs [C. C. Lee et al., "Generation of cDNA Probes Directed by Amino Acid Sequence: Cloning of Urate Oxidase", Science, 239, pp. 1288-91 (1988)]. In this case, two sets of oligonucleotides based on the known amino-terminal amino acid sequence of urate oxidase were synthesized. One set of oligonucleotides was sense strands and the other set was anti-sense strands. The sense oligonucleotides hybridized to the non-coding strand of urate oxidase cDNA present in a mixture of cDNAs. Then both sense and anti-sense oligonucleotides were used to amplify the urate oxidase cDNA. As a result, the amount of urate oxidase double-stranded

cDNA was specifically amplified, permitting ease of cloning as compared with standard cDNA library screening techniques employing similar oligonucleotide probes.

The PCR technique has also been used to amplify sequences outside the boundaries of known sequences [T. Triglia et al., "A Procedure for In Vitro Amplification of DNA Segments that Lie Outside the Boundaries of Known Sequences" Nucleic Acids Research, 16, p. 8186 (1988)]. In this application of PCR, termed "inverted PCR" (IPCR), a piece of DNA containing both known and unknown DNA sequences is circularized. The circular DNA is then linearized by cleavage at a site that lies within the boundaries of the known sequences. Oligonucleotide primers corresponding to the ends of the known sequence are synthesized in opposite orientation from that used in normal PCR because the known sequence has been inverted and now flanks the unknown DNA sequence. These primers are then used to amplify the unknown sequences.

Despite these developments, PCR technology is still limited because the oligonucleotide primers must be synthesized based on some specific known sequence which flanks the DNA to be amplified. [See, for example, T. L. Bugawan et al., "The Use of Non-Radioactive Oligonucleotide Probes to Analyze Enzymatically Amplified DNA for Prenatal Diagnosis and Forensic HLA Typing", Bio/Technology, 6, pp. 943-47 (1988).] Therefore, different oligonucleotides must be synthesized every time a different sequence is amplified. Moreover, PCR cannot be employed in situations where no sequence information is available.

Such applications, however, are desirable for a number of purposes, including the identification of previously unknown infectious agents. The causes of

many dis ases that affect humans, and other animals are unknown. It has been hypothesized that diseases, such as insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), Kawasaki disease, rheumatoid arthritis and juvenile rheumatoid arthritis, are caused by highly infective viruses or microorganisms that are present in the patient in subdetectable amounts [G. T. Horn et al., "Allelic Sequence Variation of the HLA-DQ Loci: Relationship to Serology and to Insulin-Dependent Diabetes Susceptibility, Proc. Natl. Acad. Sci. USA, 85, pp. 6012-16 (1988); H. Kikuta et al., "Epstein-Barr Virus Genome-Positive T Lymphocytes in a Boy with Chronic Active EBV Infection Associated with Kawasaki-Like Disease", Nature, 333, pp. 455-57 (1988); R. B. Sayetta, "Theories of the Etiology of Multiple Sclerosis: A Critical Review", J. Clin. Immunol., 21, pp. 55-70 (1986); E. P. Reddy et al., "Amplification and Molecular Cloning of HTLV-1 Sequences from DNA of Multiple Sclerosis Patients, Science, 243, pp. 529-33 (1989); P. E. Phillips, "Evidence Implicating Infectious Agents in Rheumatoid Arthritis and Juvenile Rheumatoid Arthritis", Clin. Exp. Rheum., 6, pp. 87-94 (1988)]. Accordingly, the need exists for a technique by which unknown infectious agents can be identified and studied, i.e., a technique which can be used to amplify any desired nucleotide sequence regardless of whether its sequence or those sequences flanking it are known.

SUMMARY OF THE INVENTION

The present invention solves the problems referred to above by providing sequence-independent gene amplification methods. These methods allow for the amplification of any isolated double stranded DNA segment even in the absence of information regarding

its nucleotide sequence or the sequence of its flanking regions. The methods of this invention are especially useful in producing clonable quantities of DNA or cDNA when such amounts are not available in nature. The present invention also provides methods for amplifying any isolated nucleic acid fragment. The invention also relates to "universal oligonucleotide primer pairs" which are used in the sequence-independent gene amplification methods of this invention.

The methods of this invention are advantageously used when about 100 molecules or less of one or more target DNA sequences are initially available. The universal oligonucleotide primer pairs of the present invention are ligated onto the ends of the DNA fragment to be amplified to create specific targets for PCR. In this manner, analyzable and clonable quantities of DNA may be synthesized.

As will be appreciated from the disclosure to follow, the universal oligonucleotide primer pairs of this invention and the methods which employ them may be used to amplify any double-stranded DNA segment or group of DNA segments. These methods produce useful quantities of the segment(s) faster and at less cost than conventional methods. The methods of this invention are useful in a wide variety of situations such as amplifying unknown nucleic acid sequences from a low titer of an infective agent, producing useful quantities of cDNA initially synthesized from trace amounts of tissue and directly producing useful amounts of cDNA or DNA from an isolated recombinant clone.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a photograph of an agarose g l on which target DNA sequenc s amplified by a method of this invention have been el ctrophoresed.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for amplifying nucleic acid molecules whose nucleotide sequence is unknown. The methods of this invention are especially useful in amplifying nucleic acid sequences which are present in minute amounts. The invention also relates to pairs of complementary oligonucleotides which are useful in the methods of this invention.

Throughout the specification and in the claims the terms "universal primer pairs", "primer pairs" and "linker pairs" refer to the complementary oligonucleotide pairs of this invention and are used interchangeably. The individual single-stranded oligonucleotides which make up the primer pairs are referred to as "primer pair members" or "linker pair members". Throughout the specification and in the claims, the DNA sequence to be amplified is referred to as "target DNA". The term "linkered target DNA" as used in the present specification and in the claims refers to the product of a ligation between the universal primer pairs of this invention and the target DNA. The term "biological sample" refers to any tissue, organ or body fluid sample obtained from any animal, especially a human. And, the term "patient" refers to any animal, particularly a human being.

The universal primer pairs of this invention may range in length from about 12 to about 40 nucleotides. They are preferably about 20 nucleotides in length. Each member of the primer pair is substantially equal in length and at least 70% complementary to the other member of the pair. Most preferably, the individual members of the primer pair are exactly th same length and are 100% complementary.

There are no specific nucleotide sequence requirements for the universal primer pairs of the present invention. However, it should be understood that certain structural requirements should be considered in order for the primer pairs to be most useful in the methods of this invention. The structural factors that should be considered in designing the primer pairs include the melting temperature of the primer pair, the amount of self-complementarity within each individual member of the pair, the state of phosphorylation of the 5' ends of the primer pair members and the length of the individual members of the pair. Each of these factors will have an effect on the efficiency of the amplification reaction, which, in turn, will effect the maximum amount of DNA that may be produced by the methods of the invention.

There is, however, one situation in which a specific nucleotide sequence requirement may exist. If the amplified, linkered target DNA is to be subsequently inserted into a vector such that the recombinant DNA molecule codes for a fusion protein (e.g., in the unique EcoRI site of the β-galactosidase gene of lambda gt11) it is preferable that neither primer pair member encode a stop codon in any translational reading frame. The existence of an in-frame stop codon in the transcribed fusion protein DNA strand will ultimately prevent translation of the fusion protein. Since the correct reading frame of the target DNA cannot be determined prior to amplification, the elimination of stop codons in all reading frames of each primer pair member avoids the problem referred to above.

Of all the above factors, the melting t mperature is probably the most important. The

melting temperature of the primer pair is the temperature at which hydrogen bonds, which normally hold the pair together, are destroyed. Above this temperature, the individual pair members separate and exist in solution as single-stranded DNA. To be effective in the sequence-independent amplification methods of this invention, the primer pair should have a melting temperature lower than the melting temperature of the target DNA sequences to be amplified. Most preferably, the melting temperature of the primer pair will be below about 70°C.

Several factors contribute to the melting temperature of the primer pair, as well as to the melting temperatures of all complementary DNA sequences. These factors are well known to those of skill in the art and include the length of the DNA sequence, the G-C content of the DNA sequence and the ionic strength of the solution containing the primer pair.

For example, the melting temperature of the universal primer pairs of the present invention may be calculated by assigning a 4°C contribution to each G-C base pair, a 2°C contribution to each A-T base pair and a 0°C or negative contribution to each mismatched base pair present in the oligonucleotide primer pair. It will be understood by those of skill in the art that the position of mismatched base pairs in the primer pairs of the present invention (i.e., 5', 3' or middle) which are not 100% complementary will also effect the melting temperature.

According to a preferred embodiment of this invention, the sequence of the linker pair is selected from the group consisting of:

NANB 15 5'AGCAGGCAGAAGTATGCAAA 3'
NANB 16 3'TCGTCCGTCTTCATACGTTT 5',

NANB 19 5'TAAGTAGGTGAGTAAGTGAG 3'
NANB 20 3'ATTCATCCACTCATTCACTC 5', and

PCR 01 5'GTTATTGCGGCCGCTTATTG 3'
PCR 02 3'CAATAACGCCGGCGAATAAC 5'.

Most preferably, the primer pair is PCR 01/PCR 02. The melting temperature of PCR 01/PCR 02 is about 60°C.

Another factor important in choosing primer pairs for use in the methods of the present invention is the absence of 3' self-complementarity. As used herein, the term "self-complementarity" refers to the ability of one molecule of an individual primer pair member to hydrogen bond to a second molecule of the same primer pair member to form double-stranded DNA. Self-complementarity is most often characterized by the presence of a restriction endonuclease site in the sequence of the oligonucleotide primer pair member.

When self-complementarity exists at the 3' end of a primer pair member, the annealing of two molecules of that primer pair member creates an appropriate substrate for DNA polymerase (i.e., a template and a primer). Because primer pair members are present in great excess over target DNA in the methods of the present invention, the above undesirable substrates rapidly consume the deoxynucleoside triphosphate molecules, resulting in drastically reduced efficiency of target DNA amplification.

Self-complementarity at either the 5' end or in the middle of a primer pair member is not detrimental to the methods of this invention. The double-stranded DNA molecule formed by the annealing of two molecules of a primer pair member displaying such self-compl mentarity is not a substrate for DNA polymerase. As used throughout the specification and

in the claims, the term "significant self-complementarity" refers to that type of self-complementarity which would favor the annealing of individual molecules of a primer pair member to one another so as to create a DNA polymerase substrate.

Finally, to be most effective in the methods of the present invention, the 5' ends of the primer pair should be non-phosphorylated. The absence of phosphorylation prevents the ligation of the linker pairs or individual primer pair members to each other. A structure which consists solely of concatamers of oligonucleotide primer pairs would create a substrate for DNA polymerase during the melting and reannealing steps of the methods of this invention. Such a reaction is undesirable because it dramatically decreases the efficiency of the amplification reaction.

The oligonucleotide primer pairs of this invention may be synthesized by any standard method known in the art. Preferably, both first and second strands of the primer pair are synthesized separately, using an automated oligonucleotide synthesizer.

The methods of the present invention require the presence of double-stranded target DNA in order to carry out amplification. Once the target DNAs have been isolated, the next step is to ligate on the primer pair. Because the amplification methods of the present invention require the ligation of the primer pair onto the target DNA by blunt-end ligation, the target DNA should first be rendered blunt-ended. This may be achieved by any conventional method, including the use of restriction enzymes which produce blunt ends, as well as by single-strand specific exonucleases which generate blunt ends from overhangs previously produced by restriction enzymes, or enzymes used in the synthesis of cDNA.

It will be obvious that the need for blunt-ending is obviated if the target DNA is initially present as a blunt-ended molecule. The optimum concentration of primer pair used in the ligation reaction will range from about 1 femtomole to about 1 picomole/μl of reaction mix. In addition, the concentration of primer pair is ideally many-fold greater than that of the target DNA.

Ligation may be performed by any number of conventional techniques. Because the primer pair is not phosphorylated, the products of the ligation will consist of a first primer pair member covalently attached to the blunt 5' terminus of the target fragments and the second primer pair member annealed to the first primer pair member via hydrogen bonding, but not covalently attached to the 3' terminus of the target DNAs.

Because ligation is achieved via blunt-ends, the primer pairs may be oriented in either direction at either end of the target DNAs, resulting in four possible combinations:

```
(1)      5'(top)3'------------------- 5'(top)3'
     3'(bottom)5' -------------------3'(bottom)5'

(2)  5'(bottom)3'------------------- 5'(top)3'
        3'(top)5' -------------------3'(bottom)5'

(3)      5'(top)3'------------------- 5'(bottom)3'
     3'(bottom)5' -------------------3'(top)5'

(4)  5'(bottom)3'------------------- 5'(bottom)3'
        3'(top)5' -------------------3'(top)5'
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wherein ------ represents the target DNA; (top) and (bottom) represent the two different members of the primer pair; 5' and 3' represent the orientation of the

primer pair and ~ represents a covalent phosphodiester bond between the target DNA and the 3' ends of the primer pair. The actual amplification step of the methods of this invention is initiated by adding an excess quantity of only one member of the primer pair. Those of skill in the art will recognize that only one of the above four molecules may actually be amplified (either (2) or (3) depending on whether the "top" or "bottom" primer pair member is used for initiation of amplification). This is of little consequence, however, since the amount of target DNA increases exponentially during each amplification step.

After the ligation has been completed, the unbonded member of the primer pair is melted off and replaced by an identical sequence that is covalently bonded to the target DNAs. This is preferably achieved by raising the temperature of the solution containing the linkered target DNA to a point at which the unbonded pair member melts off and then performing a 3' extension reaction. The temperature of the solution should not, however, be raised high enough to cause a melting of the strands of the target DNAs. It is therefore desirable to heat the solution to a temperature above the melting temperature (T_m) of the primer pair, but below the T_m of the target DNA. It will also be understood by those of skill in the art that the ionic strength of the solution may also affect the T_m. Although the T_m of the target DNA will be undeterminable in practice, the T_m of most DNA sequences of significant length (i.e., greater than about 400 base pairs) is given by the following formula:

$$T_m = 69.3 + 0.41 \text{ (mole \% G+C)}$$

Accordingly, the T_m of most target DNAs will be at least 75°C. It is therefore preferable to use a prim r pair

that has a melting temperature below about 70°C and to effect the melting of the primer pair by heating to about 72°C. The melting reaction is essentially complete after about 5 minutes.

Once the unbonded pair member is melted off, the resulting 5' overhangs are repaired by standard 3' extension means. This involves contacting the sample with a mixture of four deoxynucleotide triphosphates (dATP, dCTP, dTTP and dGTP) and a DNA polymerase, preferably a thermostable polymerase, such as Taq. The extension reaction is preferably performed at about 72°C. The 3' extension reaction is preferably carried out in the presence of an excess amount of the one of the members of the primer pair that will be used to initiate PCR. Although the added primer pair member does not participate in the 3' extension reaction, it is preferably included at this step in preparation for the subsequent polymerase chain reaction step. Alternatively, the primer pair member may be added following the 3' extension reaction. The amount of primer pair member added at this step is at least 10-fold greater and preferably at least 20-fold greater than the amount of primer pair added in the ligation reaction. This will allow subsequent amplification of the linkered target DNA in the polymerase chain reaction steps that follow.

In a more preferred embodiment, the melting off and 3' extension steps are performed simultaneously and in the presence of an excess amount of one of the primer pair members. This is achieved by adding the primer pair member, the DNA polymerase and the four deoxynucleotide triphosphates prior to or during the melting off step.

PCR is initiated following completion of the extension r action by adding an effective amount of a

primer pair member, an effective amount of a DNA polymerase and an effective amount of the four different nucleotide triphosphates. According to a preferred embodiment, the PCR reaction utilizes the primer pair member, deoxynucleotide triphosphates and DNA polymerase already present in the samples from the 3' extension step. First, the sample is heated to a temperature above the T_m of the target DNA. Preferably, the sample is heated to about 93°C to insure complete melting of the linkered target DNA strands. Additionally, the duration of this heating should preferably be kept to a minimum effective time, most preferably about 2 minutes. The sample is then cooled, preferably to about 40°C, to allow hybridization of the excess added primer pair member to its complementary sequence present on the linkered target DNA. Preferably, this step will also be complete in about 2 minutes. The sample is then reheated to a temperature which promotes primer extension, resulting in an effective doubling of the amount of properly primed target DNAs. The temperature at which primer extension is performed must be lower than the T_m of the target DNAs and, when Taq polymerase is employed, is preferably about 72°C. The duration of the primer extension step will vary depending on the length of the target DNA. The duration of the primer extension step is approximated at about 1 minute per kilobase length of target DNA. In most situations, the primer extension reaction is completed in about 4 to about 6 minutes. The melting, annealing and primer extension steps are then repeated as many times as is necessary to produce a desired amount of target DNA. These steps or their equivalents, which result in the amplification of target DNA, are collectively referred to as "PCR

means". The amount of target DNA produced may be estimated by the formula:

$$X_o 2^n$$

where X_o equals the amount of amplifiable target DNA sequence initially present and n equals the number of PCR cycles performed.

The techniques for initially obtaining the target DNA will vary depending upon the nature of the original sample. For example, if the target DNA is a collection of cDNAs synthesized from a small tissue sample or a single cell, the first step is the isolation of mRNA from that sample. This technique is well known in the art. As used herein the term "small tissue sample" refers to an amount of a biological sample which does not contain enough mRNA to produce clonable quantities of cDNA. Small tissue samples are exemplified by biopsy samples.

After the mRNA has been isolated, first- and second-strand cDNA synthesis is performed, preferably employing a cDNA synthesis kit (e.g., BRL cDNA Synthesis System, Bethesda Research Laboratories, Bethesda, Maryland). The sequence-independent gene amplification methods of this invention may then be used to produce clonable amounts of the resultant cDNA molecules. Restriction enzyme site-containing linkers or polynucleotide linkers may then be ligated onto the ends of the cDNAs following the amplification reaction. Preferably, the oligonucleotide primer pair will itself contain an internal restriction site, located either in the middle or at the 5' end, obviating the need for additional linkers.

In an alternate embodiment of the present invention, the original sample to be amplified consists of nucleic acid sequences obtained from a suspected infectious agent which is present in an intractable

titer in the infected h st. In the case of suspected enveloped virus, a biological sample containing the presumptive causative agent is obtained, homogenized if necessary, and treated with a nuclease, preferably micrococcal nuclease, to digest the host's nucleic acid sequences. The nuclease is then inactivated by any of a number of well known procedures including, but not limited to, heating, addition of metal chelaters, such as EGTA, and extraction with organic solvents, such as phenol. The causative agent's nucleic acid sequences are then isolated.

Isolation of nucleic acid sequences from the causative agent initially involves destruction of the viral envelope. This may be achieved with a variety of reagents including organic solvents, preferably phenol, lipases, and proteases. The nucleic acids may then be isolated by standard techniques. Most preferably, inactivation of the nuclease and dissolution of the viral envelope are achieved in a single step by phenol extraction. The isolated nucleic acid sequences remain in the aqueous phase which may be used directly in the methods of this invention or following precipitation and concentration. It should be obvious that precipitation of the small amount of nucleic acid sequences may be facilitated by the addition of a carrier molecule. Most preferably, the carrier molecule is a mixture of short, random sequence oligonucleotides.

It is well known that viral nucleic acid sequences may be in the form of single-stranded or double-stranded DNA or RNA. Additionally, the nucleic acid sequences may be circular or linear. In order to amplify the nucleic acid sequences from an infectious agent without first identifying their structure it is preferable that double-stranded cDNA be synthesized

from the isolated nucleic acid sequences. Because cDNA synthesis requires single-stranded nucleic acid sequences as templates, the isolated nucleic acid sequences should initially be denatured. Boiling the isolated nucleic acid sample is most preferable because it will denature both linearized and nicked, circular double-stranded nucleic acids, while having no deleterious effect on single-stranded nucleic acids. If the viral nucleic acid exists as a covalently closed double-stranded DNA circle, the boiling step is likely to introduce random nicks into each strand at low frequency. These nicked molecules will then denature. Therefore, this treatment insures the production of single-stranded nucleic acids no matter what the nature of the starting material. cDNA synthesis may then be achieved from the resulting single-stranded nucleic acids by methods which are known to those of skill in the art. The double-stranded cDNA is an appropriate target DNA for the methods of this invention. It should also be understood that isolated nucleic acids that are known to exist as linear double-stranded DNA molecules may be amplified directly by the methods of this invention without the need for synthesizing linear double-stranded cDNA.

Still another embodiment of the present invention relates to amplifying DNA inserts contained within an isolated recombinant clone. This aspect of the invention is especially useful when the target DNA is cloned into a vector, such as a bacteriophage, which cannot be replicated readily into large quantities. This method surprisingly allows those of skill in the art to produce useful quantities of these DNA inserts without the need for subcloning into a more suitable vector.

In this embodiment of the invention, the vector containing the DNA insert desired to be amplified is isolated from the host which harbors it. Such methods are well known and are commonly used in the art. The vector is then cleaved with a restriction enzyme which will release the inserted target DNA from the vector. The released target DNA and the remaining vector may then be amplified in solution together and subsequently separated on the basis of size (invariably the vector will be substantially larger than the target DNA).

In order that this invention may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting this invention in any manner.

EXAMPLE 1

Synthesis Of An Oligonucleotide Primer Pair And Preparation Of Target DNA

We first synthesized the following oligonucleotide primer pair by automated oligonucleotide synthesis:

PCR 01 5'GTTATTGCGGCCGCTTATTG 3'

PCR 02 3'CAATAACGCCGGCGAATAAC 5'.

The individual oligonucleotide primer pair members, PCR 01 and PCR 02, were synthesized separately on an automated Applied Biosystems 380A DNA synthesizer. Following synthesis, I deprotected the oligonucleotides by adding 4 ml of concentrated ammonium hydroxide to the vial containing the oligonucleotide. I incubated the vial for 8 hours at 60°C with constant stirring. I then allowed the vial to cool and transferred th contents to two 2.5 ml Eppendorf tubes. The solution in the Eppendorf tubes

was then evaporated to dryness in a Speed-Vac Concentrator (Savant, Hicksville, NY).

I further purified the individual oligonucleotide primer pair members by electrophoresis in a 10 well, 1.5 mm thick, 20% polyacrylamide gel containing 7 M urea. I dissolved the contents of one Eppendorf tube in 40 µl of 7 M urea and loaded 10 µl of the mixture into each of four wells in the gel. The samples were then electrophoresed for approximately 2 hours at 500 volts. I then removed the gel from the glass plates and wrapped it in plastic wrap. The electrophoresed oligonucleotide was visualized under ultraviolet light using a fluorescent TLC plate as a background (Whatman, Maidstone, Kent, England). Bands containing the oligonucleotide were excised from the gel and placed into an Eppendorf centrifuge tube.

The oligonucleotide was then eluted from the gel slice by adding just enough 10 mM triethylammonium bicarbonate, pH 7.6, to cover the excised gel slice and incubating at 50°C for 3 hours. The eluted material was then loaded onto a 1 ml disposable C18 column (J. T. Baker, Phillipsburg, NJ). The column had been previously washed with 10 ml of HPLC grade acetonitrile followed by 10 ml of H_2O. After loading the sample onto the column and collecting the eluate the column was washed with 10 ml of H_2O. The oligonucleotide was then eluted with 4 x 1 ml of 20% acetonitrile in H_2O. The DNA eluted in the first two 20% acetonitrile fractions as determined spectrophotometrically at 260 nm. The fractions containing the oligonucleotide were concentrated to dryness in a Speed-Vac Concentrator. The oligonucleotide was then dissolved in 1 ml of H_2O. The final concentration of oligonucleotide was determined by measuring the absorbance at 260 nm. The oligonucl otide solution was

stored at -20°C until used. The final yield of both PCR 01 and PCR 02 was 1.4 OD_{260} units (64 µg).

The target DNAs used to test the method of this invention were HincII fragments of the plasmid RSD (PCT patent application WO 88/00831). Specifically, we digested 10 µg of RSD DNA in 200 µl of appropriate restriction buffer with 40 units of HincII (New England Biolabs, Beverley, MA). The digestion was performed at 37°C for 2 hours. The digestion yielded five fragments of 4042, 3062, 2304, 1855 and 1108 base pairs. The test fragments were then diluted 1:500 into H_2O.

EXAMPLE 2

Sequence Independent Target DNA Amplification

We ligated 10 picomoles of unphosphorylated linker pair PCR 01/PCR 02 to 1 ng of test fragment. Specifically, we added 10 µl of diluted test fragments which were produced according to Example 1 to 100 µl of 1X T4 DNA ligase buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgSO_4$, 20 mM DTT, 1 mM ATP) containing 40 units of T4 ligase and 10 picomoles each of PCR 01 and PCR 02. The ligation mixture was allowed to incubate for 16 hours at 15°C.

Following ligation, we added 1 µl of the ligation mix to 100 µl of 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 100 µg/ml gelatin) containing 20 nmoles of dXTPs, 20 pmole of PCR 01 and 1 unit of Taq polymerase. The solution was then overlayed with 100 µl of sterile mineral oil and incubated at 72°C for five minutes. This incubation allowed the simultaneous melting off of the unligated member of the primer pair and repair of the resultant 20 base overhang by a 3' extension reaction.

We initiated the polymerase chain reaction by denaturing the linkered target DNA for 2 minutes at

93°C. This was followed by a cooling to 40°C for 2 minutes, which allowed the excess added PCR 01 to hybridize to its complementary sequence contained in the linkered target DNA sequence. Primer extension was carried out by raising the temperature to 72°C for 6 minutes. We repeated this 10 minute cycle 59 times. The entire cycle was performed automatically using a DNA Thermal Cycler (Perkin-Elmer/Cetus)

To show that either member of the primer pair may be effectively utilized to amplify a target DNA we performed the identical procedure described above except we substituted 20 pmoles of PCR 02 for PCR 01 in the 1X PCR buffer.

We analyzed 10 μl of each reaction by electrophoresis on a TAE/ethidium bromide/1% agarose minigel. The samples were electrophoresed at 100 volts until the dye front neared the bottom of the gel. Figure 1 displays the results of this analysis. Figure 1, lane 1 depicts 0.5 μg of unamplified test fragments. Figure 1, lanes 2 and 3 depict the 1/10th of the product of amplifying 10 picograms of test fragments for 60 cycles using PCR 01 or PCR 02, respectively, as primers.

It should be noted that only the three smallest test fragment were effectively amplified. I believe that this inefficiency can be attributed to single-stranded nicks that were present in the starting test fragments. The occurrence of such nicks is random and therefore the larger the fragment, the greater the chance of it containing a nick. When these nicks are present in a target DNA molecule one end of that molecule will be unlinkered after the first cycle of denaturation. Such a structure cannot be primed at the unlinkered end and hence, is unamplifiable.

EXAMPLE 3

Amplification Of Unknown Sequences

Insulin-dependent diabetes mellitus (IDDM) is a disease whose cause is unknown. The suspected causative agent may be a virus present at an intractable titer [G. T. Horn et al., Proc. Natl. Acad. Sci. USA, 85, pp. 6012-16 (1988)]. The methods of this invention are applied to a blood sample of a patient developing IDDM to detect and analyze the nucleic acids of this suspected virus.

A sample of blood is collected from a patient recently diagnosed as having IDDM. The blood is collected with a large bore needle into anticoagulant-free Vacutainers (Becton-Dickinson, Rutherford, NJ). Cells and fibrin clots are removed by centrifuging at 2,000 rpm for 20 minutes at 4°C. One ml of the serum is treated with 10 μg of micrococcal nuclease (Pharmacia, Piscataway, New Jersey) for 1 hour at 37°C to digest any free nucleic acid that may be contained in the serum. Nucleic acids from the suspected enveloped virus are liberated into solution by adding 5 μl of 1M DTT and 1000 units of RNasin (Promega, Madison, Wisconsin) and then extracting twice with phenol.

The aqueous phase is collected and 1 μl of synthetic primer, $pd(N)_6$, (at a concentration of 50 OD_{260} units/ml; Pharmacia) is added. The synthetic primer, $pd(N)_6$, is used to randomly prime first strand cDNA synthesis as well as serving as a carrier during subsequent precipitation of the rare viral nucleic acids. The nucleic acids are then ethanol precipitated, dried and redissolved in 30 μl of H_2O. The sample is then denatured by boiling for 3 minutes.

Renaturation is prevented by quickly chilling the boiled sample on ice.

Double-stranded cDNA is then synthesized from the denatured nucleic acids using a cDNA kit (Bethesda Research Laboratories, Bethesda, Maryland) following the manufacturer's instructions. The resulting cDNA is isopropanol precipitated, redissolved and then ethanol precipitated. The ethanol precipitate is dissolved in 20 μl of 1X T4 DNA ligase buffer containing 2 picomoles each of PCR 01 and PCR 02 and 8 units of T4 ligase. Ligation is achieved by incubating the sample overnight at 15°C.

To amplify the linkered target cDNA, 10 μl of the ligation mixture is added to 100 μl of 1X PCR buffer containing 20 nmoles of dXTPs, 20 pmoles of PCR 01 and 1 unit of Taq polymerase. The sample is overlayed with 100 μl of sterile mineral oil and heated to 72°C for 5 minutes to melt off the unligated primer pair member and repair the overhang. The linkered target cDNA is then amplified in a 60 cycle program, each cycle consisting of denaturing at 93°C for 2 minutes, primer annealing at 40°C for 2 minutes and polymerase extension at 72°C for 6 minutes.

Once amplified the linkered cDNAs are digested with NotI, which cleaves in the middle of the PCR 01/PCR 02 primer pair, and ligated into a NotI-digested expression vector. Individual clones are plated and screened with antisera obtained from a patient suffering from IDDM.

While we have hereinbefore represented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the processes of this invention. Th refore, it will be appreciated that the scope of this invention is to be defin d by the claims

appended hereto rather than the specific embodiments which have been presented hereinbefore by way of example.

CLAIMS

I claim:

1. An oligonucleotide primer pair for amplifying a double-stranded target DNA sequence, consisting of:

a first single-stranded DNA oligonucleotide primer pair member comprising a DNA sequence of between about 12 nucleic acids and about 40 nucleic acids and displaying no significant self-complementarity; and

a second single-stranded DNA oligonucleotide primer pair member comprising a DNA sequence substantially equal in length and substantially complementary to said first single-stranded DNA member; said primer pair being characterized in that said first primer pair member and said second primer pair member are not phosphorylated and the T_m of said oligonucleotide pair is less than about 70°C.

2. The oligonucleotide primer pair according to claim 1, wherein said first primer pair member and said second primer pair member both consist of 20 nucleic acids.

3. The oligonucleotide primer pair according to claim 2, said primer pair being selected from the group consisting of NANB 15/NANB 16, NANB 19/NANB 20 and PCR 01/PCR 02.

4. The oligonucleotide primer pair according to claim 1, wherein said primer pair is further characterized by the presence of a restriction endonuclease site.

5. The oligonucleotide according to claim 4, wherein said primer pair is PCR 01/PCR 02.

6. A method for amplifying a double-stranded target DNA sequence in a sample, comprising the step of ligating onto said target DNA the oligonucleotide primer pair according to any of claims 1 to 5.

7. The method according to claim 6, comprising the subsequent steps of:

(a) heating said target DNA to a temperature between the T_m of said oligonucleotide primer pair and the T_m of said target DNA to melt off the unbonded oligonucleotide primer pair member so as to create 5' overhangs; and

(b) repairing said 5' overhangs by 3' extension means.

8. The method according to claim 7, wherein said 3' extension means are performed in the presence of an effective amount of a first oligonucleotide primer pair member, an effective amount of a thermostable DNA polymerase, and an effective amount of four different nucleotide triphosphates.

9. The method according to claim 7, wherein an effective amount of a first oligonucleotide primer pair member, an effective amount of a thermostable DNA polymerase, and an effective amount of four different nucleotide triphosphates are added to said sample prior to or during said heating of said target DNA.

10. The method according to claim 7 comprising the subsequent step of amplifying said target DNA by PCR means.

11. The method according to claim 10 wherein said PCR means comprise the steps of:

(a) heating said sample containing said target DNA to a temperature above the T_m of said target DNA so as to separate the strands;

(b) cooling said sample to a temperature effective to promote hybridization of said first oligonucleotide primer pair member to a complementary DNA sequence present in said separated target DNA strands produced in step (a);

(c) maintaining said sample at an effective temperature and for an effective amount of time to promote the activity of said thermostable DNA polymerase and to synthesize an extension product of said first oligonucleotide primer pair member which is complementary to said separated strand produced in step (a); and

(d) repeating steps (a), (b) and (c) for an effective number of times.

12. The method according to claim 6, wherein said target DNA is double-stranded cDNA.

13. The method according to claim 12, wherein said double-stranded cDNA is synthesized from messenger RNA present in a small tissue sample or in a single cell.

14. The method according to claim 12, wherein said double-stranded cDNA is synthesized from

the isolated nucleic acid sequences of an infectious agent present in a biological sample.

15. The method according to claim 14, comprising the initial steps of:

(a) digesting host nucleic acid sequences contained in said biological sample;

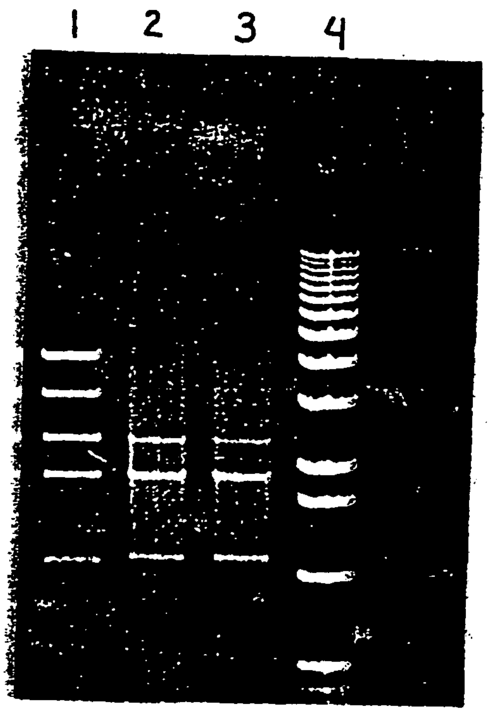
(b) inactivating the reagent used to perform said digestion;

(c) isolating said nucleic acid sequences of said infectious agent; and

(d) denaturing said nucleic acid sequences of said infectious agent under conditions which create an effective template for cDNA synthesis.

16. The method according to claim 15, wherein said biological sample is obtained from a patient suffering from a disease selected from the group consisting of insulin-dependent diabetes mellitus, Kawasaki disease, multiple sclerosis, rheumatoid arthritis and juvenile rheumatoid arthritis.

17. The method according to claim 16, wherein said disease is insulin-dependent diabetes mellitus.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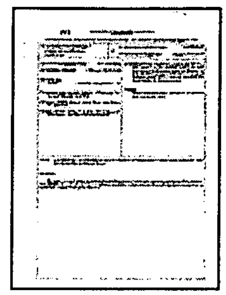
Title: WO9009457A2: OLIGONUCLEOTIDE PRIMER PAIRS FOR SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHICH EMPL



Country: /O World Intellectual Property Organization (WIPO)

Kind: **A2** Publ.of the Int.Appl. without Int.search REP. (See also: WO9009457A3)

Inventor: **ASEK, Mark, P.;**

Assignee: **BIOGEN, INC.**
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Priority Number: eb. 14, 1989 **US1989000310763**

Abstract:

The present invention relates to methods for amplifying isolated nucleic acid sequences, particularly DNA sequences, whose nucleotide sequences are unknown. The invention also relates to oligonucleotide primer pairs that may be employed in the amplification methods of the present invention.
[Show in French]

INPADOC Legal Status: how legal status actions

Designated Country: AT AU BE CA CH DE DK ES FR GB IT JP LU NL SE

Family:

PDF	Patent	Pub. Date	Filed	Title
	WO9009457A3	Oct. 4, 1990	Feb. 14, 1990	OLIGONUCLEOTIDE PRIMER PAIRS F SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHI(EMPLOY THEM
	WO9009457A2	Aug. 23, 1990	Feb. 14, 1990	OLIGONUCLEOTIDE PRIMER PAIRS F SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHI(EMPLOY THEM
	EP0458909A1	Dec. 4, 1991	Feb. 14, 1990	OLIGONUCLEOTIDE PRIMER PAIRS F SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHI(EMPLOY THEM
	CA2046919AA	Aug. 15, 1990	Feb. 14, 1990	OLIGONUCLEOTIDE PRIMER PAIRS F SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHI(EMPLOY THEM
	AU5404890A1	Sept. 5, 1990	Feb. 14, 1990	OLIGONUCLEOTIDE PRIMER PAIRS F SEQUENCE INDEPENDENT GENE AMPLIFICATION AND METHODS WHI(EMPLOY THEM

5 family members shown above

Description:

Expand full description

+ OLIGONUCLEOTIDE PRIMER PAIRS FORSEQUENCE INDEPENDENT GENE AMPLIFICATIONAND METHODS WHICH EMPLOY THEM
+ TECHNICAL FIELD OF INVENTION
+ BACKGROUND ART
+ SUMMARY OF THE INVENTION
+ BRIEF DESCRIPTION OF THE DRAWINGS
+ DETAILED DESCRIPTION OF THE INVENTION

First Claim:

Show all claims

lo An oligonucleotide primer pair foramplifying a double-stranded target DNA sequence,c of:a first single-stranded DNAoligonucleotide primer pair member comprising a DNAsequen between about 12 nucleic acids and about 40nucleic acids and displaying no significant self-complementarity; anda second single-stranded DNAoligonucleotide primer pair membe comprising a DNAsequence substantially equal in length andsubstantially-complementary t single-stranded DNA member; said primer pair beingcharacterized in that said first primer p member andsaid second primer pair member are not phosphorylatedand the T, of said oligonucleotide pair is less thanabout 700C.2o The oligonucleotide primer pairaccording to wherein said first primer pairmember-and said second primer pair member both consistof 2 acids.3a The oligonucleotide primer pairaccording to claim 2, said primer pair being selecte group consisting of NANB 15/NANB 16, NANB19/NANB 20 and PCR OI/PCR 02*4* The oligonucleotide primer pairaccording to claim 1, wherein said primer pair isfurther character the presence of a restrictionendonuclease site.PCt/US-90/00866

Forward References:

PDF	Patent	Pub.Date	Inventor	Assignee	Title
	US5422252	1995-06-06	Walker; George T.	Becton, Dickinson and Company	Simultaneous amplit multiple targets